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13. ABSTRACT *(Maximum 200 Words)*

Proteins containing a Caspase-Associated Recruitment Domain (CARD) have previously been shown to serve as key regulators of tumor cell survival as well as regulators of other cellular processes, such as cytokine production. Interleukin-1 beta (IL-1B) is a cytokine which has been found to be expressed in breast cancer cells and may be associated with more aggressive and invasive breast tumors. Through a bioinformatics approach, we identified a novel CARD protein which also contained a nucleotide binding domain (NACHT) and a region of leucine-rich repeats (LRR). Here we report the cloning and functional characterization of CLAN (CARD, LRR, And NACHT-containing protein). CLAN was found to be expressed in several breast cancer cell lines as well as in monocytes by RT-PCR. Co-immunoprecipitation studies revealed that the CARD of CLAN associated with the CARD of several other proteins including caspase-1, Nod2, and NAC. When assayed using an IL-1B ELISA, CLAN was found to induce the activation of caspase-1. Through its interactions with other CARD-containing proteins, CLAN may regulate the survival of breast cancer cells and could be utilized as a novel anti-tumor target or diagnostic/prognostic biomarker.

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Introduction

Proteins containing a Caspase-Associated Recruitment Domain (CARD) have previously been shown to serve as key regulators of tumor cell survival as well as regulators of other cellular processes, such as cytokine production. Interleukin-1 beta (IL-1β) is a cytokine which has been found to be expressed in breast cancer cells and may be associated with more aggressive and invasive breast tumors (Jin *et al.*, 1997; Kurtzman *et al.*, 1999). Through a bioinformatics approach, we identified a novel CARD protein which also contained a nucleotide binding domain (NACHT) and a region of leucine-rich repeats (LRR). Here we report the cloning and functional characterization of NAC-X or CLAN (CARD, LRR, And NACHT-containing protein). NAC-X was found to be expressed in several breast cancer cell lines as well as in other human tissues by RT-PCR. Co-immunoprecipitation studies revealed that the CARD of NAC-X associated with the CARD of several other proteins including caspase-1, Nod2, and NAC. When assayed using an IL-1β ELISA, NAC-X was found to induce the activation of caspase-1. Through its interactions with other CARD-containing proteins, NAC-X may regulate the survival of breast cancer cells and could be utilized as a novel anti-tumor target or diagnostic/prognostic biomarker.

Body

Specific Aim 1:

Specific Aim 1 of this research project was to determine the expression pattern of NAC-X in normal and malignant mammary tissues as well as in normal human tissues. Utilizing RT-PCR, it was determined that this gene was expressed in a number of normal tissues including lung, brain, colon, prostate, spleen, and leukocytes [figure 1]. RT-PCR also demonstrated NAC-X expression in several breast cancer cell lines including MDA MB231, MDA MB435, and MCF-7 (data not shown). Northern blotting using a NAC-X-specific probe revealed the expression of a 3.5-kb transcript primarily in lung tissue. Only faint expression was seen in other tissues using Northern analysis, most likely due to assay insensitivity.

The next task of Specific Aim 1 was to clone the full length NAC-X cDNA. To accomplish this goal, RACE (Rapid Amplification of cDNA Ends) PCR was utilized. Gene-specific primers corresponding to regions within the NAC-X genomic DNA prediction were used in conjunction with a common universal primer to amplify the full 5' and 3' regions of the NAC-X mRNA from lung and liver cDNA. These experiments resulted in the cloning of the full length NAC-X gene (now designated CLAN-A, for CARD, LRR, And NACHT-containing protein) as well as several smaller alternatively spliced isoforms of this gene (designated CLAN-B, -C, and –D). The final task of Specific Aim 1 was to create a specific polyclonal antibody for the NAC-X gene for use in measuring protein expression in breast tumor specimens. The first attempt to generate an antibody using a polypeptide from the NAC-X sequence has failed and a second attempt is currently underway.

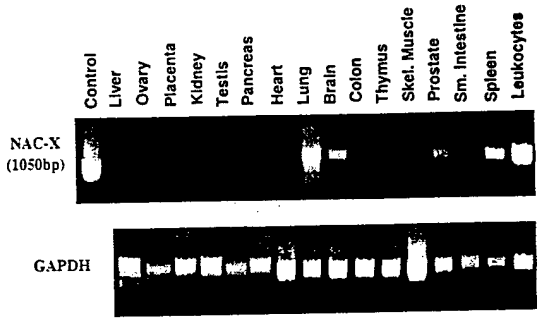

Figure 1. Analysis of NAC-X expression in normal human tissues. RT-PCR analysis of NAC-X expression in a cDNA panel derived from various tissues using NAC-X-specific primers or primers specific for GAPDH to control for RNA integrity.

Specific Aim 2:

The second Specific Aim of this project was to evaluate the associations of NAC-X with other CARD-containing proteins. Using transient transfection and co-immunoprecipitation assays, it was determined that the CARD domain of NAC-X interacts with the CARD domains of caspase-1, Nod2, NAC, and with its own CARD domain. Further screening using additional CARD-containing proteins (CARDIAK, caspase-5, Nod1, Apaf-1, caspase-9, RAIDD) revealed no other associations by co-immunoprecipitation. Transient transfection of epitope-tagged NAC-X demonstrated that the full-length protein also interacts with full length caspase-1, Nod2 and NAC [caspase-1 shown in figure 2].

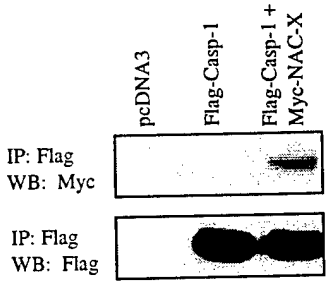

Figure 2. NAC-X co-immunoprecipitates with caspase-1. 293T cells were transfected with control vector (pcDNA3), with flag-tagged caspase-1, or with both caspase-1 and myc-tagged full length NAC-X. Following immunoprecipitation and 12% PAGE, proteins were detected using monoclonal antibodies and ECL.

To examine the oligomerization of NAC-X through its NACHT domain (formerly called NB-ARC domain), GST fusion proteins were generated, but with little success. These results were probably to improper folding of the expressed domain in bacteria and its subsequent relegation to inclusion bodies. As an alternative method, transient transfection and overexpression of epitope-tagged NACHT domains were used in co-

immunoprecipitation assays. These experiments determined that NAC-X was able to oligomerize through self-association of its NACHT domain [figure 3].

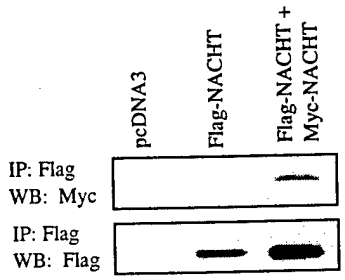

Figure 3. Oligomerization of NAC-X NACHT domain. 293T cells were transfected with control vector (pcDNA3), with flag-tagged NAC-X NACHT domain, or with both flag-tagged and myc-tagged NAC-X NACHT domain. Following immunoprecipitation and 12% PAGE, proteins were detected using monoclonal antibodies and ECL.

Specific Aim 3:

The goal of Specific Aim 3 was to determine the specific steps in the apoptotic pathway regulated by NAC-X. This aim was based on the prediction that NAC-X, due to its homology with Apaf-1, would bind and regulate proteins involved in the intrinsic apoptotic pathway. Transfection of NAC-X expression plasmids into 293T cells along with plasmids encoding either BAX or Fas revealed that NAC-X had no significant effect on either of these apoptosis pathways (using DAPI staining of apoptotic nuclei). Since NAC-X was found to bind Caspase-1, the influence of this gene on caspase-1 function was then investigated. Co-transfection of NAC-X and caspase-1 expression plasmids resulted in an increase in caspase-1-mediated apoptosis in 293T cells. Since the main function of caspase-1 in most cell types is believed to be the regulation of processing of cytokines such as IL-1β and not the regulation of apoptosis, this endpoint was then analyzed using further transfection experiments. In these studies, caspase-1 activity was monitored by measuring the amount of secreted (processed) IL-1β in the supernatant of transfected cells using an ELISA assay. It was determined that NAC-X functions as an enhancer of caspase-1 activity and thus most likely plays a role *in vivo* in regulating the secretion of this cytokine [figure 4]. Since several studies have suggested that IL-1β may be associated with a more aggressive and invasive breast tumor phenotype, NAC-X may affect the survival of tumors by upregulating the production of this cytokine as opposed to directly affecting the intrinsic apoptosis machinery.

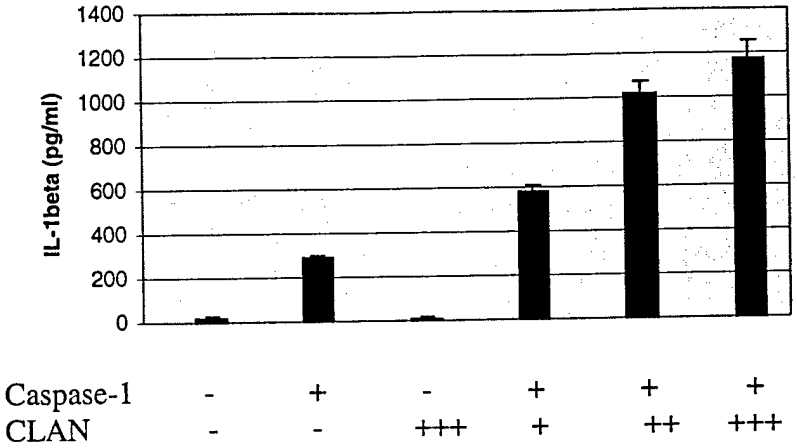

Figure 4. Effects of CLAN on IL-1beta secretion. 293T cells were transiently transfected with the indicated expression plasmids and were assayed for IL-1beta secretion 24hr later using ELISA.

Specific Aim 4:

To investigate the *in vivo* role of NAC-X in tumor cell survival, experiments utilizing dominant negative constructs and antisense oligonucleotides in breast cancer cell lines will be performed. Expression constructs and antisense oligonucleotides are currently being generated as a basis for these studies. Further experiments utilizing SCID mouse xenograft models will also help to elucidate the importance of NAC-X as a survival regulator and diagnostic marker for breast cancer *in vivo*.

Key Research Accomplishments

- Expression pattern of NAC-X in human tissues has been determined.
- Full length NAC-X and several alternatively spliced isoforms have been cloned.
- Several CARD-containing interaction partners have been found for NAC-X, most significantly caspase-1.
- NAC-X was found to self-associate, or oligomerize through its CARD and NACHT domains.
- NAC-X was discovered to be an activator of caspase-1 and a regulator of IL-1β secretion.

Reportable Outcomes

Manuscript (completed before award start date): Damiano JS, Stehlik C, Pio F, Godzik A, Reed JC. CLAN, a novel human CED-4-like gene. *Genomics* 75(1-3):77-83, 2001.

Conclusions

This investigation has led to the cloning and characterization of a novel gene involved in the regulation of caspase-1. Although caspase-1 can be involved in regulating apoptosis in different cell types, it differs functionally from the other caspases in that it is also capable of mediating cytokine release (IL-1 and IL-18) by cleaving the pro forms of these proteins. NAC-X (CLAN) is novel among most known regulators of caspase-1 because it induces, rather than suppresses, the action of this protease. IL-1β, which has been correlated to a more aggressive and invasive breast tumor phenotype, may therefore be controlled by NAC-X. Other evidence has linked IL-1β to the activation of ER-α, meaning this cytokine may modulate hormonal activity in breast tumors (Spiers *et al.*, 1999). This function may make NAC-X an attractive target for novel drug or protein inhibitors for use in breast cancer therapy. The observation that NAC-X oligomerizes through its NACHT domain also makes this protein an ideal candidate for drug discovery as this function has the potential to be antagonized by modified nucleotide analogs. Additionally, if linked to breast tumor survival or invasiveness, expression levels of NAC-X in tumor cells may prove to be a useful diagnostic tool in evaluating treatment options based on predicted level of disease aggressiveness.

References

Jin L, Yuan, RQ, Fuchs A, Yao Y, Joseph A, Schwall R, Schnitt SJ, Guida A, Hastings HM, Andres J, Turkel G, Polverini PJ, Goldberg ID, Rosen Em. Expression of interleukin-1beta in human breast carcinoma. Cancer 80(3):421-34, 1997.

Kurtzman SH, Anderson KH, Wang Y, Miller LJ, Renna M, Stankus M, Lindquist RR, Barrows G, Kreutzer DL. Cytokines in breast cancer: IL-1alpha and IL-1beta expression. Oncol. Rep. 6(1):65-70.

Spiers V, Kerin MJ, Newton CJ, Walton DS, Green AR, Desai SB, Atkin SL. Evidence for transcriptional activation of ERalpha by IL-1beta in breast cancer cells. Int. J. Oncol. 15(6):1251-4, 1999.